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In Vitro Shoot Multiplication of Elite Sugarcane (Saccharum officinarum L.) Genotypes Using Liquid Shake Culture System

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Abstract
Study was carried out with an objective to determine the appropriate concentrations and combinations of 6-benzylaminopurine (BAP) and kinetin for in vitro shoot multiplication of elite sugarcane genotypes i.e., N52 and N53. Shoot tip was used as explant source. Shoot initiation from explant of the two genotypes was achieved at a combination of BAP, kinetin and NAA (0.5 mg/l each). For shoot multiplication, the regenerated and twice subcultured shoots on semi-solid medium were transferred on liquid Murashige and Skoog medium containing 3% sucrose, fortified with various concentrations and combinations of BAP (0, 0.5, 1, 1.5, 2 mg/l) and Kinetin (0, 0.5, 1, 1.5 mg/l).The cultures were agitated continuously on an orbital shaker moving at 80 rpm. Analysis of variance (ANOVA) showed that the interaction effects of 6-benzylaminopurine, kinetin and the sugarcane genotypes on number of shoots per explant, average shoot length and number of leaves per shoot was very highly significant ($P < 0.0001$). Genotype N52 showed a maximum of 6.95 ± 0.19 shoots per explant with 4.75 ± 0.06 cm shoot length and 5.65 leaves per shoot on liquid MS medium fortified with 2 mg/l BAP + 0.5mg/l kinetin while genotype N53 produced a maximum of 6.30 ± 0.26 shoots per explant with 3.94 ± 0.03 average shoot length and 5.83 leaves per shoots on liquid MS medium supplemented with 1.5 mg/l BAP + 0.5 mg/l kinetin. Finally, from study results we can deduce that the application of this protocol helps for rapid in vitro shoot multiplication of elite sugarcane genotypes.
Keywords: Murashige and Skoog, shoot tip, explant, 6-benzylaminopurine

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous perennial plant that belongs to the family *Gramineae* (Jahangir and Nasir, 2010). It is octoploid (2n=80) having x=10 basic chromosome number (Aitken *et al*., 2010) and is the most photosynthesizer, C_4 plant (Jalaja *et al*., 2008). Sugarcane is widely cultivated as commercial crop in all tropical and subtropical regions in over 100 countries of the world situated between 37°N and 31°S (Hunsigi, 2001). According to FAOSTAT (2014), sugarcane is cultivated on 26.1 million hectares producing 1.83 billion tons of cane on worldwide. While in Ethiopia, it is cultivated on 22,389 hectares producing 2.7 million tons of crushable stem.

Sugarcane is a multipurpose industrial cash crop and main source of raw materials for the production of sugar. It accounts for nearly 70 percent of centrifugal sugar produced globally (Sengar *et al*., 2010) while the rest is produced from sugar beet. Besides sugar, agricultural and industrial by-products of sugar production processes are widely used for animal nutrition, food processing, ethanol and biogas production, paper manufacturing, steam power generating and fertilizer. Furthermore, sugar manufacture is an indispensable industry in contributing to the national economy particularly in providing employment opportunity to nation and foreign exchange earnings (Jalaja *et al*., 2008).

Commercially, sugarcane is propagated vegetatively via stem cuttings with each cutting or sett having two or three buds. This conventional propagation method has low propagation rate usually one to ten in one year period of time. Hence, once a superior genotype is selected, it usually takes 8-10 years to produce adequate quantity of seed material that enable to plant the newly released genotype at large commercial scale. During that period, yield potential of the genotype starts to decline due to infestation of different systemic diseases during the multiplication stage in the open field (Khan and Rashid, 2003). This method also requires large nursery space (Behera and Sahoo, 2009) and planting materials (40 - 60,000 setts/ha) (Hunisgi, 2001), which is laborious and time consuming (Pandey *et al*., 2011), consequently, increase cost of production. Thus the high demand for newly released genotypes could not be fulfilled in time through conventional propagation methods. Therefore, it is imperative to employ efficient propagation system that realizes mass multiplication of newly evolved sugarcane genotypes in a short period of time.

Plant tissue culture is nowadays the most viable and successful technique that offers the best methodology through micropropagation to ensure high quantity and quality of sugarcane planting material within a short period of time and space (Sengar *et al*., 2011). It has become a solely alternative to conventional propagation method. In contrast to conventional methods where one bud produces 4-5 shoots, tissue culture can produce around 260,000 healthier and identical plants from a single shoot tip in 3-4 months (Hendre *et al.,* 1983).

In line with this, several protocols have been developed for rapid multiplication of newly released and commercially important genotypes of sugarcane through shoot tip, callus, and axillary bud cultures (Baksha *et al*., 2002; Cheema and Hussain, 2004; Ali *et al*, 2008). However, slow rate of bud proliferation, high cost of agar and labor make conventional micropropagation costly (Prasad and Gupta, 2006). Besides, agar creates potential problems such as non-uniform distribution of nutrients and growth regulators in the medium (Muhammad *et al*., 2007).

One way of overcoming these problems is the use of liquid culture system instead of semisolid media. Accordingly, several investigators suggested the use of this approach **for fast and efficient micropropagation of sugarcane genotypes (Kenganal *et al*., 2009; Khan *et al.,* 2009; Pathak *et al*., 2009). However, g**enotype specific protocols are needed as the hormonal requirements for *in vitro* morphogenetic responses vary from genotype to genotype in sugarcane **(Ramanad and Lal, 2004)** and reports are scarce on liquid culture in sugarcane genotypes of Ethiopia. Hence, this study was conducted with the objective of determining the appropriate concentrations and combinations of BAP and kinetin plant growth regulators for *in vitro* shoot multiplication of elite sugarcane genotypes through liquid culture.

MATERIALS AND METHODS

The study was conducted at the National Agricultural Biotechnology Laboratory of the Ethiopian Agricultural Institute of Research, in Holetta. It is located 28 km west of Addis Ababa, Oromia region.

Plant Materials

The study was conducted using two pipeline sugarcane genotypes N52 and N53. These genotypes were selected based on their yield performance and sugar quality. N52 yields 206.3 ton cane/ha with 15.36 % sucrose content while N53 gives 166.9 ton cane /ha with 13.36% sucrose content. The materials were obtained from the Ethiopian Sugar Corporation, Wonji.

Mother plant propagation

The seedcane (setts) with two buds were treated with hot water at 50^{o}C for 2 hours followed by immersing in fungicide (Bayleton® DF 50%) solution at rate of 1 g/l for 5 minutes. The treated setts were planted in plastic pots containing mixture of autoclaved forest soil, farmyard manure and river sand in the ratio of 1:1:1 and allowed to grow in a screen house of Holetta National Agricultural Biotechnology Laboratory for five months.

Explant sterilization and preparation

Actively growing shoot tops were taken from 5-months-old screen house grown healthy mother plants and used as explants. Trimmed shoot tops were taken to the laboratory and washed thoroughly under running tap water for 30 minutes and the size reduced to 10 cm length by cutting off at the two ends. The explants were further washed for 30 minutes with tap water containing a drop of liquid detergent solution plus two drops of tween-20 with continuous shaking and rinsed three times with double distilled water. Later, the explant was taken to a laminar air flow cabinet and immersed in 0.1% (w/v) Bavistin® DF 50% (Carbendizem) fungicide solution, ascorbic acid (0.2% w/v) and citric acid (0.4% w/v) for 30 minutes followed by three times rinsing each for five minutes with sterile double distilled water. The shoot tips were washed again with 70% ethanol for one minute and rinsed with sterile double distilled water three times each for five minute to remove residual ethanol from the shoot tip surface. Finally, the explants were surface sterilized with 50% (v/v) aqueous solution of Sodium hypochlorite (5.25% w/v active chlorine) containing a few drops of a wetting agent (tween-20) with gentle shaking for 25 minutes. After pouring out sodium hypochlorite solution, the explants were rinsed with sterile double distilled water three times each for five minutes to remove all the trace of the sterilant. Subsequently, about 1.5 cm long shoot tip explants were aseptically excised from sterilized segments.

Culture Establishment

The explants were placed in test tube containing 20 ml of culture media containing MS basal salt fortified with BAP, Kinetin and NAA (0.5 mg/l each) plant growth regulators and 20 g/l sucrose as carbon source and solidified with 4.5g/l of agar. The pH was adjusted to 5.8 prior to autoclaving at 121^{o}C and 1.05kg/cm^{3} for 20 minutes. The test tubes with cultured explants were properly plugged with non-absorbent cotton, sealed with PVC film. Afterwards, the cultures were transferred and maintained on shelves in growth room adjusted at temperature of 25±2^{o}C under 16 hours photoperiod with photo flux density of 30 µmol m^{2}/ s provided by cool white fluorescent light and 70-80% relative humidity.

In vitro Shoot Multiplication

Healthy micro-shoots having the same size obtained from the initiation stage were used for shoot multiplication experiment after maintaining on plant growth regulator free semi-solid medium to minimize the carry over effect of the initiation medium. These micro-shoots were transferred to full MS liquid media supplemented with 3% sucrose. The media also supplemented with different concentrations and combinations of BAP (0, 0.5, 1.0, 1.5 & 2 mg/l) and kinetin (0, 0.5, 1.0 & 1.5 mg/l). The pH of the media was adjusted to 5.8 and autoclaved at 121˚C and 1.05kg/cm^{3} for 20 minutes. Each treatment was replicated four times and five shoots were cultured in each culture jar. The culture jars were properly sealed with PVC film, and randomly placed on a rotary shakers that

revolved at a speed of 80 rpm and the shakers were maintained for 30 days in environmentally controlled growth room shelves at 25˚C ± 1˚C and 16 and 8 hrs light and darkness, respectively. The experiment was laid out in CRD with three factor factorial combination (2x5x4 factorial arrangement). Sub-culturing was carried out fortnightly on the same medium. Data on number of shoot per explants, length of shoots and number of leaves per shoot were carefully recorded after four weeks.

Data Analysis

The analysis of variance (ANOVA) appropriate for design was performed to detect the significance of differences among the treatment means and the treatment means were compared using REGWQ (Ryan-Einot-Gabriel-Welsch) at a 5% probability level using a statistical software program SAS version 9.2 (SAS Institute Inc., 2009).

RESULTS AND DISCUSSION

The result showed that shoot multiplication was influenced by the effect of both genotypes and growth regulators. Analysis of Variance (ANOVA) revealed that interaction effect of genotype, BAP and kinetin was highly significant (P<0.001) on the number of shoots per explant, shoot length, and number of leaves per shoot (Table 1). The interaction of genotype, BAP and kinetin indicated that all the three factors are reliant on each other for *in vitro* shoot proliferation of sugarcane.

Table 1. Analysis of variance (ANOVA) for shoot multiplication of the two genotypes with different concentration of BAP and Kinetin growth regulators

Source of variation	DF	Number of shoots per explant	Shoot length (cm)	Number of leaves per shoot
		MS	MS	MS
Genotype	1	14.28***	11.70***	11.18***
BAP	4	17.66***	8.85***	8.32***
Kinetin	3	13.56***	12.30***	13.53***
Genotype*BAP	4	4.74***	0.24***	0.73***
Genotype*Kinetin	3	0.88***	0.19***	0.023*
BAP*Kinetin	12	8.81***	3.66***	10.24***
Genotype*BAP*Kinetin	12	4.97***	0.38***	1.00***
CV %		5.55	5.43	4.20

*** = very highly significant at P ≤ 0.0001, * = significant at P ≤ 0.05, NS = Non-significant at P ≥ 0.05, DF = Degree of freedom, BAP = 6- Benzyl aminopurine, MS = mean square CV = Coefficient of variation

The result of the different combination and concentrations of BAP (0, 0.5, 1, 1.5, 2 mg/l) and Kinetin (0, 0.5, 1, 1.5 mg/l) on the shoot multiplication is presented in Table 2. Shoot multiplication was not observed within 4 weeks when explant cultured on MS medium devoid of plant growth regulators (BAP and kinetin) in both genotypes (Table 2). However, increasing the concentration of kinetin alone from 0 to 0.5 mg/l resulted in 3.35 and 3.15 shoots per explant for N52 and N53, respectively. In the same way, increasing BAP concentration alone from 0 to 0.5 mg/l gave 4.80 and 3.25 shoot per explant in genotype N52 and N53, respectively. This result indicates that the supplementation of exogenous plant growth regulators to MS medium is imperative to develop multiple shoots. In fact, cytokinins are capable of overcoming apical dominance and release lateral buds from dormancy thereby enhance shoot multiplication (George et al., 2008).

In the present study, it was observed that the two genotypes responded differently to the same media for all parameters studied. A significantly highest average number of shoots per explant (6.95) was observed in genotype N52 on MS medium fortified with 2 mg/l BAP in combination with 0.5 mg/l kinetin (Table 2 and Figure 1a) while N53 gave only 3.20 shoot per explant on the same medium composition. Similarly, N53 produced maximum of 6.30 shoots per explant on MS medium fortified with 1.5 mg/l BAP + 0.5 mg/l kinetin (Table 2 and Figure 1b) while the same medium composition resulted in only 5.15 shoots per explant in N52.

Table 1: The effect of BAP and Kinetin on number of shoots per explant, shoot length and number of leaves per shoot

PGRs(mg/l)		Genotypes					
		N52			N53		
BAP	Kinetin	Number of shoots per explant ±SD	Shoot length (cm) ±SD	Number of leaves per shoot ±SD	Number of shoots per explant	Shoot length (cm) ±SD	Number of leaves per shoot
0	0	0.00^{s} ± 0.00	0.00^{s} ± 0.00	0.00^{q} ± 0.00	0.00^{s} ± 0.00	0.00^{s} ± 0.00	0.00^{q} ± 0.00
	0.5	3.35^{m-q} ± 0.25	3.67^{b-h} ± 0.23	3.83^{l-o} ± 0.24	3.15^{pq} ± 0.19	2.87^{n-q} ± 0.03	4.83^{cf} ± 0.10
	1	4.15^{jk} ± 0.19	3.63^{b-i} ± 0.04	4.58^{e-j} ± 0.24	3.45^{l-q} ± 0.19	3.08^{l-p} ± 0.09	4.93^{cf} ± 0.10
	1.5	3.80^{l-n} ± 0.16	3.20^{i-p} ± 0.10	4.55^{e-j} ± 0.17	3.75^{k-o} ± 0.19	2.79^{o-q} ± 0.13	5.18^{c-d} ± 0.22
0.5	0	4.80^{e-h} ± 0.16	3.87^{b} ± 0.17	3.45^{op} ± 0.13	3.25^{n-q} ± 0.25	2.99^{l-p} ± 0.15	5.67^{ab} ± 0.17
	0.5	3.95^{j-l} ± 0.19	3.91^{b} ± 0.22	3.70^{m-p} ± 0.18	5.15^{d-f} ± 0.19	3.31^{e-n} ± 0.08	4.75^{d-f} ± 0.06
	1	3.45^{l-q} ± 0.19	4.55^{a} ± 0.27	3.95^{k-n} ± 0.19	4.50^{g-j} ± 0.26	3.86^{b} ± 0.03	4.48^{f-j} ± 0.10`
	1.5	3.25^{n-q} ± 0.10	3.66^{b-i} ± 0.59	3.83^{l-o} ± 0.17	4.40^{i-j} ± 0.33	3.50^{b-i} ± 0.25	3.95^{k-n} ± 0.06
1	0	5.00^{e-g} ± 0.28	3.64^{b-i} ± 0.22	5.25^{bc} ± 0.27	3.50^{l-q} ± 0.26	2.77^{q} ± 0.14	4.70^{ef} ± 0.18
	0.5	6.05^{bc} ± 0.19	3.71^{b-e} ± 0.16	5.63^{abc} ± 0.19	3.60^{k-p} ± 0.28	2.88^{n-q} ± 0.35	4.98^{e-e} ± 0.17
	1	3.75^{k-o} ± 0.25	3.60^{b-j} ± 0.22	4.00^{k-n} ± 0.18	5.60^{cd} ± 0.16	3.35^{e-l} ± 0.01	4.65^{e-h} ± 0.06
	1.5	3.50^{l-q} ± 0.12	3.28^{g-n} ± 0.08	3.28^{p} ± 0.25	3.05^{pq} ± 0.10	3.24^{g-o} ± 0.18	4.58^{e-j} ± 0.28
1.5	0	5.15^{d-f} ± 0.19	3.45^{d-l} ± 0.02	4.38^{g-k} ± 0.29	4.45^{g-j} ± 0.19	2.50^{q} ± 0.03	4.70^{ef} ± 0.22
	0.5	5.15^{d-f} ± 0.25	3.39^{d-l} ± 0.11	4.93^{c-f} ± 0.26	6.30^{b} ± 0.26	3.94^{b} ± 0.03	5.83^{a} ± 0.10
	1	6.35^{b} ± 0.19	3.27^{g-n} ± 0.24	4.60^{e-l} ± 0.14	3.60^{k-p} ± 0.43	2.98^{m-p} ± 0.06	5.20^{c-d} ± 0.22
	1.5	3.85^{k-m} ± 0.34	3.24^{g-o} ± 0.03	3.93^{k-n} ± 0.22	2.15^{r} ± 0.19	2.46^{n} ± 0.19	4.60^{e-l} ± 0.25
2	0	5.35^{de} ± 0.19	3.20^{h-p} ± 0.08	3.60^{n-p} ± 0.18	4.65^{f-i} ± 0.25	2.30^{r} ± 0.01	4.23^{h-l} ± 0.22
	0.5	6.95^{a} ± 0.19	4.75^{a} ± 0.06	5.65^{ab} ± 0.21	3.20^{oq} ± 0.28	3.82^{b-d} ± 0.03	5.75^{a} ± 0.10
	1	4.50^{g-j} ± 0.26	4.66^{a} ± 0.20	4.13^{j-m} ± 0.17	3.05^{pq} ± 0.19	3.74^{b-e} ± 0.19	4.58^{e-j} ± 0.15
	1.5	3.40^{l-q} ± 0.16	3.68^{b-g} ± 0.17	3.93^{k-n} ± 0.10	3.00^{q} ± 0.16	3.16^{j-p} ± 0.11	4.18^{i-l} ± 0.05
CV%		5.55	5.48	4.23	5.55	5.48	4.23

Values in the same column and with different letters are significantly different from each other according to REGWQ at P<0.05

Regarding shoot length, N52 produced the highest shoot length (4.75 cm) with the maximum number of leaves per shoot (5.65) on MS medium supplemented with 2 mg/l BAP + 0.5 mg/l Kinetin while only 3.07 shoot length with 4.23 leaves per shoot produced in N53. This different growth response to the same media composition might be as a result of difference in inherent endogenous growth hormone level among genotypes (George *et al*, 2008). Earlier research reports also confirmed that different genotypes respond differently to PGR and other media components (Khan and Rashid, 2003; Khan *et al*., 2006).

It is apparent from Table 2 that the use of 2 mg/l BAP alone produced only 5.35 shoots per explant, which was increased to 6.95 shoots by addition of 0.5 mg/l kinetin in genotype N52. In the same way, increased shoot number per explant was observed in genotype N53 from 4.45 to 6.30 when 0.5 mg/l kinetin was added to MS medium containing 1.5 mg/l BAP. This positive effect indicates the significance of adding the two growth regulators in combination rather than alone in shoot multiplication medium. In similar experiments, proliferation of higher number of shoots per explant due to the synergistic effect of the two cytokinins (BAP and Kinetin) was reported (Khan *et al*., 2009; Adilakshmi *et al*., 2014). Ali and Afghan (2001) also reported that medium supplemented with BAP and Kinetin resulted in rapid multiplication of shoots.

The best result achieved in genotype N53 is consistent with the result obtained by Khan *et al*. (2009). They reported a maximum multiplication from HSF-240 produced 11 shoots per explant; 16.5 cm mean shoot length, and 32 leaves per shoot on MS medium amended with 1.5 mg/l BAP + 0.5 mg/l kinetin. The current result in N52 is also in harmony with the results reported by Mekonen *et al*. (2014), who obtained best result from Co 678 genotype on MS medium fortified with 2 mg/l BAP + 0.5 mg/l Kinetin with 9.1 number of shoots 6.83cm shoot length and 5.67 leaves per shoot. In both cases, the observed difference in number of shoots per explant, number of leaves and shoot length could be due to genotypic difference. Adilakshmi *et al*. (2014) also obtained optimum multiplication of 7.74 and 6.39 shoots per explant at MS medium augmented with lower concentration of BAP and Kinetin (0.25 mg/l BAP + 0.1 mg/l Kinetin) in genotype 96A3 and Co 6907, respectively.

Similar results were also reported by Singh (2003) who observed an average of 12.33 shoots on MS medium fortified with 1.5 mg/l BAP + 0.5 mg/l Kinetin. Khan *et al*. (2009) observed maximum shoot multiplication on MS medium augmented with 1 mg/l BAP + 0.1 Kinetin and 1 mg/l BAP + 0.5 Kinetin in sugarcane genotype CPF-237 and HSF-240, respectively. Ali *et al*. (2008) achieved best shoot multiplication for sugarcane genotype BL-4 on MS medium amended with 0.50 mg/l BAP + 0.25 mg/l kinetin. However, there are also reports (Sughra *et al*., 2014) that indicated higher multiplication rate of sugarcane at lower concentration of BAP than obtained in this study. Result of the present study indicated that 2 mg/l BAP + 0.50 mg/l Kinetin was the optimum and best hormone concentration and combination for maximum shoot multiplication of sugar cane genotype N52.While 1.5 mg/l BAP + 0.5 mg/l kinetin was found to be the best for sugarcane genotype N53.

Figure1. In vitro shoots multiplication: A) N52 at 2 mg/l BAP and 0.5 mg/l Kinetin B) N53 at 1.5 mg/l and 0.5 mg/l Kinetin

Conclusion

In the present study it was observed that a combination of 2 mg/l BAP + 0.5 mg/l Kinetin was the best combination for shoot multiplication of genotype N52 while 1.5 mg/l BAP + 0.5 mg/l kinetin was the optimum combination for genotype N53 as reflected by increased number of shoot per explant, number of leaves per shoot and length of shoots. Comparison of the two genotypes showed that N52 was a better responsive than N53 for *in vitro* multiplication in a liquid culture. From this study we can conclude that the developed protocol provides rapid shoot multiplication technique that enables to minimize the time required for large-scale propagation of newly evolved and high yielding sugarcane genotypes.

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